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Ionotropic Glutamate Receptors are Associated with Motoneurons in Axial White Muscle of Zebrafish (*Danio rerio*).

by

Keith Jeffrey Todd

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Physiology and Cell Biology

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Dr. Jeffrey I. Goldberg (committee member)

Sept 5/03

Abstract

Previous studies have indicated that glutamate may be involved in vertebrate neuromuscular junction (NMJ) function. Zebrafish (*Danio rerio*) were used to investigate the presence of presynaptic ionotropic glutamate receptors (iGluRs; AMPA, KA and NMDA) at the NMJ. Immunostaining of NMDA receptor subunits (NR2A) colocalized with immunostaining of primary motoneuron axons. Patch-clamp recordings of miniature endplate currents (mEPCs) from axial white muscle were performed. Local perfusion of the NMJ suggested a presynaptic action of iGluR agonists. NMDA and AMPA-induced increases in mEPC frequencies were blocked with AP-5 (50 μM) and GYKI 52466 (50 μM) respectively. Further investigation revealed no effect of the kainate receptor-specific antagonist Sym 2801 (10 μM). Kainate-induced rises in mEPC frequency were blocked with the AMPA receptor-specific antagonist GYKI 52466. Application of glutamate (1 mM) with the glutamate uptake inhibitor TBOA (200 μM) significantly increased mEPC frequency. Results suggest that AMPA and NMDA receptors are associated with motoneuron axons of larval zebrafish.

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List of Abbreviations

ACh = acetylcholine

AChR = acetylcholine receptor

AMPA = α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

AMPAR = α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor

AP-5 = D-(-)-2-amino-5-phosphonopentanoic acid

ATP = adenosine triphosphate

CaM KII = calcium/calmodulin-dependent protein kinase type II

CaP = caudal primary motoneuron

CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione

CNS = central nervous system

Curare = d-tubocurarine

DMSO = dimethyl sulfoxide

dpf = days post-fertilization

EC_{50} = 50% excitatory concentration

EGTA = Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

GluR1-4 = AMPAR subunit types 1-4

GluR5-7 = low affinity KAR subunit types

GTP = guanosine triphosphate

GYKI 52466 = 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine

HEPES = 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

hpf = hours post-fertilization

iGluR = ionotropic glutamate receptor

I/V = current/voltage relationship

KA = kainic acid

KA1-2 = high affinity KAR subunit types

KAR = kainic acid receptor

LTD = long-term depression

LTP = long-term potentiation

M1-4 = receptor transmembrane regions 1-4

MEPC = miniature endplate current

MiP = middle primary motoneuron

MS-222 = tricaine methane sulfonate

nAChR = nicotinic acetylcholine receptor

NMDA = N-methyl-D-aspartate

NMDAR = N-methyl-D-aspartate receptor

NMJ = neuromuscular junction

NO = nitric oxide

NR1 = NMDAR subunit type 1

NR2A-D = NMDAR subunit type 2A-D

PKA = protein kinase A

PKC = protein kinase C

PMN = primary motoneuron

PNS = peripheral nervous system

RoP = rostral primary motoneuron

SMN = secondary motoneuron

Sym 2081 = (2S,4R)-4-methylglutamate

TBOA = DL-threo-β-benzyloxyaspartate

TTX = tetrodotoxin

1. Introduction

1.1. Zebrafish Development

Zebrafish (*Danio rerio*) continue to gain popularity as a model for vertebrate development. Two reasons for this are their prolific reproduction and rapid development, making them an excellent model for developmental genetics. Other favourable characteristics include their transparent body and relatively simple nervous system. The simple nervous system has visually identifiable cells, which allows for *in vivo* studies (Hale et al. 2001; Kuwada et al. 1990). It is also possible to use knowledge of their developmental rate and characteristic escape behaviour for physiological and behavioural assays (Liu and Fetcho 1999). The zebrafish is arguably one of the best model systems for vertebrate development.

Zebrafish eggs are fertilized externally and develop at a rate determined by the environmental temperature. Embryos proceed through visually distinguishable developmental stages that allow for calculation of the age providing the environmental temperature is maintained. A standard 28.5° C has been established to normalize all developmental studies in zebrafish (Westerfield 1995). At this developmental rate, embryos will begin to respond to touch at about 20-26 hours post-fertilization (hpf) and hatch at about 50 hpf (Saint-Amant and Drapeau 1998). It is possible to perform detailed *in vivo* experiments on both the central nervous system (CNS) and muscular systems prior to hatching (Ali et al. 2000b; Nguyen et al. 1999).

The nervous system of zebrafish embryos begins to develop early in life. Some of the first neurons to project processes are sensory Rohon Beard cells of

the spinal cord (Kuwada et al. 1990). These neurons and others send out growth cones as early as 16 hpf. There are few differentiated cells in the spinal cord of embryos at this age. Identifiable cells include Rohon Beard cells, about four types of interneurons, and primary motoneurons (Kuwada et al. 1990). By about 4 days post-fertilization (dpf), there are about eight differentiated and identifiable interneuron types (Hale et al. 2001) and two motoneuron types (Myers et al. 1986). As spinal cord neurons are differentiating and sending out axons, so too are neurons from the hindbrain. These reticulospinal neurons send axons down the spinal cord starting at around 20 hpf, continuing to do so past 30 hpf (Mendelson 1986). At about the same time that reticulospinal neurons begin to project growth cones, axons from Rohon Beard cells reach the hindbrain. These axons terminate near to the Mauthner cell (Bernhardt et al. 1990; Mendelson 1986) and form connections to initiate a touch-mediated escape response (Liu and Fetcho 1999; Saint-Amant and Drapeau 1998). Reticulospinal neurons contact motoneuron axons to complete the circuit and initiate output of the escape response (Jontes et al. 2000; O'Malley et al. 1996). This, however, does not occur until about 30 hpf. Remodelling of the spinal cord and hindbrain continues past 15 dpf as neurons are added and removed over the course of development (An et al. 2002; Svoboda et al. 2001; Williams et al. 2000).

Two types of motoneurons are present in the spinal cord of zebrafish (Myers et al. 1986; van Raamsdonk 1983; Westerfield et al. 1986). Their differentiation and axon projection are temporally regulated through differential gene expression (Beattie et al. 1997). Primary motoneurons appear first and

project axons as early as 20 hpf (Eisen et al. 1986). These projections pass through ventral roots (Myers et al. 1986) and form bundles that project to three general regions of the axial muscle in stereotyped patterns (Eisen et al. 1986). In each hemisegment of the spinal cord there are 3 primary and about 20 secondary motoneurons (Myers et al. 1986). The 3 primary motoneurons are called rostral (RoP), middle (MiP) and caudal primary (CaP) motoneurons; they project to mid, dorsal and ventral axial musculature respectively (Myers et al. 1986). Secondary motoneurons project to all regions of the axial muscle (van Raamsdonk 1983). Motoneurons begin to fire action potentials at about 24 hpf (Saint-Amant and Drapeau 2000). Bursting of motoneurons displays characteristic patterns at different ages with the pattern changing over the first five days of development (Buss and Drapeau 2001). Initial bursting at 24 hpf to 2 dpf displays periodic depolarizations with bursts that are cyclical in amplitude (Buss and Drapeau 2001). By 4 dpf the bursting pattern has changed to one with short bursts and slow depolarizations reflecting a beat and glide behaviour (Buss and Drapeau 2001). These changes in motoneuron firing patterns are likely reflected in neuromuscular junction (NMJ) development and swimming behaviour.

There are two main types of muscle fibers in embryonic and larval zebrafish. These are red and white muscle fibers that are characterized as being slow or tonic, and fast or phasic fibers respectively (van Raamsdonk 1983; van Raamsdonk et al. 1978; Waterman 1969). Further differentiation of the muscle occurs later in life, resulting in the presence of pink fibers with intermediate

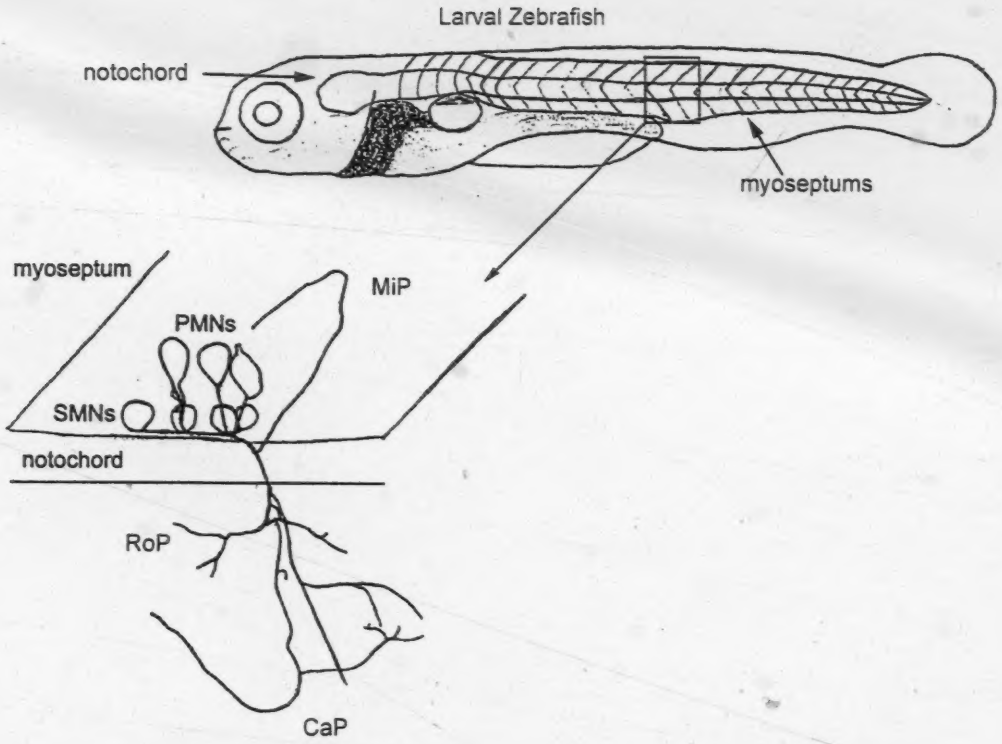


Fig 1. Schematic of larval zebrafish and motoneuron projections. Primary motoneurons (PMNs) project to dorsal (MiP), mid (RoP) and ventral (CaP) musculature. Secondary motoneurons (SMNs) project to all regions of axial muscle. Axons project in stereotyped patterns to the different regions of muscle.

properties (van Raamsdonk et al. 1982). Red and white fibers have different distributions (van Raamsdonk et al. 1978, 1982; Waterman 1969) and different firing properties (Buckingham and Ali 2003). It is believed that muscle fibers are polyinnervated (Westerfield et al. 1986) with white fibers being innervated by one primary and multiple secondary motoneurons, while red fibers may be innervated by multiple secondary motoneurons only (de Graaf et al. 1990). It has been shown that during the escape response, primary motoneurons fire first followed by activation of secondary motoneurons (Liu and Westerfield 1988). This suggests that white fibers mediate the escape response. Miniature endplate currents (mEPCs) result from the spontaneous release of one vesicle of neurotransmitter, and are present from at least 27 hpf in white muscle (Nguyen et al. 1999). The kinetics of mEPCs changes from 27 hpf to 6 dpf with currents getting larger and faster with time. This change in mEPCs is likely due to development of the NMJ, which is poorly formed at 30 hpf, but is strengthened by 3 dpf (Drapeau et al. 2001).

Many developmental changes occur in zebrafish embryos and larvae during the early stages of life. Development and differentiation of cells in the nervous system and axial musculature begins prior to 20 hpf. Although the onset of development is rapid, many changes continue to occur in the CNS (Ali et al. 2000b), peripheral nervous system (PNS; Nguyen et al. 1999) and musculature (van Raamsdonk et al. 1982) during, and in some cases, past the first week of life.

1.2. The Vertebrate Neuromuscular Junction

Neuromuscular junctions of vertebrates are largely controlled through the release of acetylcholine (ACh) into the synaptic cleft that then binds to postsynaptic nicotinic ACh receptors (nAChRs). Release of ACh is mediated by presynaptic Ca^{2+} channels, which are localized in bands closely associated with neurotransmitter active zones (Robitaille et al. 1993). Ion flow through nAChR channels consists of both monovalent (Na^+, K^+) and divalent ions (Ca^{2+}) (Sanes and Lichtman 1999). Synaptic transmission begins to occur almost immediately following contact between outgrowing motoneurons and muscle (Kidokoro and Yeh 1982). However, there are many developmental changes that occur to both the motoneuron and muscle following initial contact. These developmental changes result in strengthening of the synapse and expression of the mature phenotype.

Many of the postsynaptic developmental changes involve clustering of nAChRs and development of the endplate (Bevan and Steinbach 1977; Jessell et al. 1979). In uninnervated muscle there is relatively random and homogenous distribution of nAChRs (Anderson and Cohen 1977; Bevan and Steinbach 1977; Frank and Fishbach 1979). However, following innervation, clusters of receptors begin to form through redistribution of old nAChRs and insertion of new receptors (Anderson and Cohen 1977). These clusters of nAChRs begin to form opposite motoneuron terminals and active zones (Bevan and Steinbach 1977; Frank and Fishbach 1979) suggesting that motoneurons may be involved in the clustering phenomenon (Jessell et al. 1979). Along with clustering occurring opposite

terminals, the extrasynaptic density of nAChRs decreases with time following innervation (Bevan and Steinbach 1977). Muscle is shaped such that there are multiple, alternating troughs and crests. The clustering of nAChRs has been localized to crest regions that are close to presynaptic terminals (Flucher and Daniels 1989). The density of nAChRs at crests is approximately 1000-fold higher than at extrasynaptic regions (Frank and Fishbach 1979), with the high density dropping rapidly as distance from the endplate increases (Flucher and Daniels 1989; Goldman et al. 1988).

Another change that occurs on the postsynaptic membrane involves nAChR subunit expression. These receptors are thought to be pentameric, containing 2 α, and 1 each of β, γ, and δ subunits in the embryonic form (Mishina et al. 1986). A fifth subunit denoted ε has similar structure to the γ subunit and is thought to be expressed in nAChRs of adults (Mishina et al. 1986). Mishina and colleagues (1986) found that functional channels would only form in the presence of either ε or γ, while omission of both of these subunits resulted in non-functional channels. Maturation of postsynaptic nAChRs involves an overall down regulation of nAChR mRNA (Goldman et al. 1988) as well as the γ to ε switch (Mishina et al. 1986). Adult channels display increased single channel conductance and greater Ca^{2+} permeability (Mishina et al. 1986; Villarroel and Sakmann 1996). The level of Ca^{2+} permeability has been suggested to be important for development of the NMJ.

Signaling from motoneurons has been shown to be important for development and differentiation of muscle and nAChRs (Balice-Gordon et al.

1993; Fontaine et al. 1986; Jessell et al. 1979; Loeb and Fishbach 1995) as well as Schwann cells (Mirsky and Jessen 1996). Clustering and synthesis of nAChRs is thought to be a major target for motoneuron signaling (Fontaine et al. 1986; Jessell et al. 1979; Jones et al. 1997; Loeb and Fishbach 1995). Increased presence of nAChRs at endplate is thought to be due in part to the activity of ACh receptor-inducing activity (ARIA), also known as neuregulin (Loeb and Fishbach 1995). Neuregulin may act through a phosphorylation cascade to induce expression of the ε subunit as well as increasing votage-gated Na^+-channel expression (Loeb and Fishbach 1995). This helps to develop the mature phenotype. Another potential neuron-derived developmental factor is calcitonin gene-related peptide, which is a splice variant of the calcitonin gene (Fontaine et al. 1986). Fontaine and colleagues (1986) suggest that this gene product increases synthesis of nAChRs. Also, a neuron-derived factor known as agrin, is thought to be responsible for clustering of nAChRs (Jones et al. 1997).

Motoneuron pruning is another developmental change that may involve reciprocal signaling between Schwann cells and neurons or neurons and muscle. It is known that during motoneuron outgrowth, multiple motoneurons will innervate a given muscle fiber (Balice-Gordon et al. 1993; Hirata et al. 1997). Following early polyinnervation and the formation of multiple nAChR clusters, removal of all but one motoneuron occurs. Balice-Gordon et al. (1993) suggest that neurons may provide a maintenance factor, as nAChR clusters opposite retracting nerves are lost. It appears that pruned motoneurons retract rather than degenerate (Hirata et al. 1997), which might be mediated by Schwann cells. This

is suggested through the finding that perisynaptic Schwann cells (non-myelinating Schwann cells) are found at a 1:1 ratio at early neuron terminals. Following retraction these Schwann cells migrate to another terminal until the remaining terminal has multiple perisynaptic Schwann cells (Hirata et al. 1997).

Development of vertebrate NMJs is a complicated process that is continually being redefined. Current investigations are resulting in new cellular molecules and neurotransmitters that are involved in differentiation and maturation of pre and postsynaptic components. Although the basic function of the NMJ has been established for many years, there is still much that can be learned about synapse formation in general as well as specific aspects of NMJ development.

1.3. NMDA Receptors

Glutamate receptors are made up of both ionotropic and metabotropic glutamate receptors. Ionotropic glutamate receptors (iGluRs) contain N-methyl-D-aspartate receptors (NMDARs) and non-NMDARs. The channel and subunit structure of NMDARs differs from other iGluRs, therefore, resulting in different functional properties. These properties include voltage-dependent Mg^{2+} sensitivity, Ca^{2+} permeability and a high affinity for glutamate. NMDARs have been shown to be important in many developmental processes and synaptic plasticity (Grant and O'Dell 2001; Lee et al. 1998; Luscher et al. 2000).

NMDARs are heteromeric receptors that contain a combination of two NR1 and two NR2 subunits. These subunits have four transmembrane domains

(M1-4). There are 11 splice variants of NR1 subunits that impart different properties on channels. Further diversity is gained through selective expression of the different NR2 subunits A-D (Hollmann and Heinemann 1994). Developmental changes in subunit expression meet changing cell requirements during development (Cathala et al. 2000; Hestrin 1992; Kirson et al. 1999; Lindlbauer et al. 1998). More recently, a new class of NMDAR subunits, NR3A and B, have been described. These subunits appear to combine with NR1 and NR2 subunits to modulate NMDAR activity (Nishi et al. 2001; Perez-Otano et al. 2001). Subunit types are summarized in table I. Differences in NR2 subunits include Mg^{2+} sensitivity (Kirson et al. 1999), ligand affinity (Regalado et al. 2001), Ca^{2+} permeability (Krupp et al. 1996; Lei and McBain 2002), activation and inactivation kinetics (Lindlbauer et al. 1998; Nabekura et al. 2002; Perouansky and Yaari 1993) and mechanisms of modulation (Krupp et al. 1996; Umemiya et al. 2001). NMDARs have a relatively lower open probability *in vivo* compared to excised patches (Rosenmund et al. 1995), which is thought to be due to the presence of intracellular modulatory factors such as phosphatases (Krupp et al. 1996; Umemiya et al. 2001) and kinases (Moon et al. 1994). Magnesium blocks NMDARs at negative potentials by entering and binding to the channel pore (Mayer et al. 1984; Nowak et al. 1984). This is thought to be due in part to charged amino acids in the M2 region of the pore (Sharma and Stevens 1996).

Activation of NMDARs requires both glutamate and the coagonist glycine (Benveniste et al. 1990; Johnson and Ascher 1992; Thedinga et al. 1989). Both ligands are important for proper NMDAR gating and kinetics (Benveniste et al.

1990; Johnson and Ascher 1992; Priestley and Kemp 1994; Sircar and Zukin 1991), and in the presence of glycine, NMDARs have a higher affinity for glutamate (Priestley and Kemp 1994; Sircar and Zukin 1991). Open NMDARs allow for flux of Na^+, K^+ and Ca^{2+} and have different conductance levels (Cull-Candy and Usowicz 1987; Jahr and Stevens 1987; Premkumar et al. 1997).

Binding of glycine to NMDARs has been investigated through competitive binding studies, which established that glycine binds to multiple sites, most likely two (Clements and Westbrook 1991; Thedinga et al. 1989). Similarly, glutamate binding studies have shown that two glutamate molecules bind to NMDARs during gating (Benveniste and Mayer 1991). It is thought that glycine binding sites are found on NR1 subunits while NR2 subunits have glutamate binding sites (Hollmann and Heinemann 1994). The binding affinity of glutamate is subunit-dependent, allowing for some differences in NMDAR properties (Chen et al. 2001). Regalado et al. (2001) also demonstrated subunit-dependent modulation of glycine affinity through NR1-NR2 interactions and suggested that different NR2 subunits interact with NR1 subunits differently and alter glycine affinity through these interactions.

NMDARs are distinct excitatory amino acid receptors that are thought to be important for many different cellular processes. Their unique mechanisms of modulation and permeability to Ca^{2+} has lead them to be the focus of much research, where they are reported to be important for synaptic plasticity, neuronal development and pathologies such as excitotoxicity (Grant and O;Dell 2001; Hollmann and Heinemann 1994; Luscher et al. 2000).

Table I. Summary of ionotropic glutamate receptor subtypes and their corresponding subunits.

Receptor Subtypes	AMPA	Kainate	NMDA
Subunits	GluR1	GluR5	NR1 (11 splice variants)
	GluR2	GluR6	NR2A
	GluR3	GluR7	NR2B
	GluR4	KA1	NR2C
		KA2	NR2D
			NR3A,B

1.4. AMPA/Kainate Receptors

The other group of iGluRs consists of two receptor types, which are often grouped together due to their similar kinetics and pharmacology. These receptors are α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs) and kainic acid receptors (KARs) and are responsible for fast excitatory transmission. Until recently, these receptors have been difficult to pharmacologically isolate and are therefore often grouped together. They have been found on both pre- and post-synaptic membranes where they modulate presynaptic activity (MacDermott et al. 1999) and postsynaptic processes (Hollmann and Heineman 1994), resectively. Their kinetic properties, pharmacology and ion permeability add necessary diversity to glutamatergic synapses. AMPARs are composed of various combinations of subunits, GluR1-4 (Table I), that have four transmembrane domains (Hollmann and Heinemann 1994). AMPAR subunits combine as tetramers (Rosenmund et al. 1998), but may only bind two glutamate molecules during gating (Clements et al. 1998), and unlike NMDARs, they do not require glycine as a coligand (Hollmann and Heinemann 1994).

Although homomeric GluR2 channels are known to exist, they are not abundant and show low conductance levels. However, heteromeric channels are widespread throughout the CNS and often have greater conductance levels (Boulter et al. 1990). It is thought that the main reason for low GluR2 permeability is RNA editing to the second transmembrane (M2) region that results in replacement of a glutamine residue with a positively charged arginine

(Burnashev et al. 1996; Sommer et al. 1991). This site is referred to as the Q/R site, and replacement with arginine in GluR2 subunits renders them impermeable to Ca^{2+}, while they remain permeable but selective for monovalent cations (Burnashev et al. 1996). Other AMPAR subunits are permeable to Ca^{2+}, Na^{+} and K^{+} and may also be slightly permeable to anions (Burnashev et al. 1996).

Editing of the M2 region of AMPARs only occurs in GluR2 subunits. For this reason, GluR2 subunits are important for altering channel properties such as rectification, extracellular modification (by lectins) and ion permeability (Boulter et al. 1990; Bowie and Mayer 1995; Inglis et al. 2002; Kamboj et al. 1995; Koh et al. 1995; Liu and Cull-Candy 2002). Channels containing GluR2 subunits display linear I/V curves while other subunit compositions are inwardly rectifying (Boulter et al. 1990; Kamboj et al. 1995). The rectification of channels containing subunits other than GluR2 has been shown to be due to intracellular polyamines (Bowie and Mayer 1995; Kamboj et al. 1995; Koh et al. 1995) that may interact with the channel pore (Koh et al. 1995). Since inward rectification is often lost in outside-out patches, it is thought that polyamines are washed out. However, this can be countered with inclusion of spermine in pipette solutions (Bowie and Mayer 1995; Kamboj et al. 1995; Koh et al. 1995).

Another major form of editing that occurs with AMPAR subunits results in flip and flop variants. This is editing to the extracellular region between M3 and M4 (Sommer et al. 1990). This region impacts ligand efficacy (Watase et al. 1997), deactivation and desensitization (Koike et al. 2000; Lomeli et al. 1994; Partin et al. 1996) and affects single channel open times (Swanson et al. 1997).

In addition, another report (Lomeli et al. 1994) has indicated that glycine/arginine editing occurs in a region just before the flip/flop region. This G/R site can alter channel properties in ways that are dependent on whether the flip or flop variant is present. Multiple forms of RNA editing provide mechanisms for enhancing AMPAR channel diversity.

The most commonly discussed endogenous ligand for AMPARs is glutamate. This neurotransmitter binds to AMPARs with a relatively low affinity that is reflected in the fast on and off kinetics of these receptors (Kleppe and Robinson 1999). The binding of glutamate to AMPARs can be altered through extracellular glycosylation sites (Hoffman et al. 1998). With lectins bound to these extracellular glycosylation regions the glutamate affinity of AMPARs is increased. Further modulation of AMPARs can occur through phosphorylation and dephosphorylation reactions. Kinases that interact with GluR subunits include PKA, PKC, CaMKII and the Src family of kinases (Barria et al. 1997a, b; Derkach et al. 1999; Esteban et al. 2003; McDonald et al. 2001; Rong et al. 2001). Phosphorylation reactions on AMPAR subunits result in potentiation (Barria et al. 1997a, b; Lee et al. 2000), depression (Lee et al. 2000; Xiao et al. 2001), receptor internalization and insertion (Esteban et al. 2003; Matsuda et al. 2000; Xiao et al. 2001), and alter single channel conductance (Banke et al. 2000; Derkach et al. 1999). These mechanisms can be initiated through interactions with both NMDARs (Pickard et al. 2001) and metabotropic glutamate receptors (Xiao et al. 2001).

The other type of non-NMDARs, kainate receptors, are composed of low (GluR5-7) and high (KA1 and KA2) affinity subunits (Table I; Hollmann and Heinemann 1994). These subunits combine to form glutamate-sensitive tetramers (Hollmann and Heinemann 1994). KARs were suggested to be distinct from AMPARs through studies that found differences in location of high-affinity KA binding (Agrawal and Evans 1986; Monaghan and Cotman 1982) and later through genetic diversity (Hollmann and Heinemann 1994). Other groups have reported subtle differences in KA and AMPAR kinetics, ligand affinities and desensitization properties (Burnashev et al. 1996; Lambolez et al. 1991; Swanson et al. 1996). There are also differences in channel conductance and receptor localization (Smith and Howe 2000; Swanson et al. 1998; Wyllie et al. 1993). More recently, differences have been found in AMPAR and KAR responses to methylglutamates (Donevan et al. 1998) and other molecules (Bortolotto et al. 1999; Clarke et al. 1997; Donevan and Rogawski 1993; Swanson et al. 1998) that have allowed for pharmacological isolation and manipulation of these receptors.

1.5. Presynaptic Receptors

The presence of presynaptic receptors has been suggested for over 40 years. Dudel and Kuffler (1961) suggested the presence of these receptors in the inhibition of neurotransmitter release in crayfish. More recently, the presence of presynaptic glutamate receptors has been established (Barnes et al. 1994; Krebs et al. 1991; Liu 1997; Siegel et al. 1994). In the vertebrate CNS, presynaptic

iGluRs facilitate or enhance neurotransmission (Berretta and Jones 1996; Krebs et al. 1991; Liu et al. 1994) while they may have inhibitory actions in crustaceans (Parnas et al. 1994). Alternatively, KARs have been shown to have an indirect inhibitory action through facilitation of GABA release (Kerchner et al. 2001; Rodriguez-Moreno et al. 1997). All presynaptic receptors share a commonality in modulation of synapse function.

Glutamate receptors have been localized to presynaptic membranes in the thalamus (Liu 1997), hypothalamus (Ginsberg et al. 1995), entorhinal cortex (Berretta and Jones 1996) and dorsal roots (Liu et al. 1997; Marvizon et al. 1997). Cochilla and Alford (1997, 1999) have also, indicated the presence of presynaptic receptors in non-mammalian vertebrates. In reticulospinal neurons of the lamprey hindbrain, presynaptic NMDARs were shown to increase presynaptic Ca^{2+} and decrease synaptic failure rate (Cochilla and Alford 1999). Berretta and Jones (1996) report a similar mechanism for NMDARs in the entorhinal cortex where they act as autoreceptors to increase the release of glutamate. The presence of iGluRs presynaptically at synapses is also reported to modulate the release of substances other than glutamate. One such neurotransmitter is dopamine, which can be modulated by presynaptic NMDARs (Krebs et al. 1991). Ginsberg et al. (1995) indicate the presence of presynaptic iGluRs in oxytocin containing neurons of the hypothalamus. They hypothesized that presynaptic iGluRs might modulate oxytocin release. NMDARs are also involved in the pain pathway, pain perception and modulation of substance P release (Liu et al. 1997). These autoreceptors are thought to increase substance P release or

glutamate release and may also be involved in neurokinin receptor internalization (Liu et al. 1997; Marvizon et al. 1997). The presence of iGluRs at various synapses throught the CNS has been well documented. These receptors can modulate the release of many neurotransmitters, and may act to modulate other synaptic properties and developmental processes.

In the mid 1990's, the presence of glutamate and glutamate receptors at vertebrate NMJs was suggested (Fu et al. 1995; Waerhaug and Ottersen 1993). Since then, various studies have investigated the presence of NMDARs and glutamate in mammalian (Barthelemy-Requin et al. 2000; Berger et al. 1995; Grozdanovic and Gossrau 1998; Malomouzh et al. 2003) and non-mammalian preparations (Chen et al. 1998; Liou et al. 1996). These receptors may alter quantal (Liou et al. 1996) or non-quantal (Malomouzh et al. 2003) release of ACh and may be present on presynaptic (Fu et al. 1995; Liou et al. 1996) or postsynaptic membranes (Berger et al. 1995; Malomouzh et al. 2003). Although strong cases for the presence of NMDARs on both the pre- and post-synaptic membranes have been made, their true location remains unresolved. Another unresolved aspect of this research is the developmental expression of NMDARs. Liou et al. (1996) suggested that NMDARs might be replaced with metabotropic glutamate receptors later in life. This is somewhat supported by Pinard and colleagues (2002) who found metabotropic glutamate receptors at the NMJs of adult amphibians. Furthermore, no group has investigated the presence of non-NMDARs at NMJs. Supporting the presence of glutamate receptors at NMJs is the suggestion that glutamate can be coreleased with ACh (Vyas and Bradford

1987). Waerhaug and Ottersen (1993) have also localized glutamate to motoneuron terminals in rats. Presynaptic iGluRs at the NMJ may be involved in development or some other Ca^{2+}-mediated process (Fu et al. 1995). Although there is a growing body of evidence indicating the presence of iGluRs at NMJs, the functional and physiological significance of their presence there has yet to be determined.

1.6. Research Objectives

The zebrafish is an excellent developmental model system for studies of vertebrates. It is the purpose of this work to investigate the presence of iGluRs at the zebrafish NMJ. It is hypothesized that: 1) both NMDA and non-NMDA receptors are located at the NMJ, 2) iGluRs at the NMJ are located on the presynaptic membrane and 3) presynaptic iGluRs modulate the release of ACh. These hypotheses will be tested through the use of immunohistochemistry and electrophysiology. Intact preparations will allow for investigation of presynaptic iGluRs *in vivo*.

2. Materials and methods

2.1. Animals

All use of animals adhered to guidelines established by the University of Alberta and the Canadian Council of Animal Care. Adult fish (Carolina Biological, Burlington, NC) were spawned daily and fertilized eggs were collected and kept at 28.5° Celcius. Prior to all protocols, larval zebrafish were anesthetized in 0.02% MS-222.

2.2. Immunohistochemistry

Whole zebrafish larvae (4-6 dpf) were fixed for 2 h in 2% paraformaldehyde and permeablized with 4% Triton X-100 for 30 min. Permeablized animals were washed prior to incubation in primary antibody (zn-1, mouse anti-zebrafish 1:50, University of Oregon) and anti-NR2A (1:200 goat anti-rabbit, Calbiochem, San Diego, CA), or anti-NR2B (1:200 goat anti-rabbit, Sigma, St. Louis, MO) for 48 h at 4° on a rotary shaker. Zn-1 antibodies recognize primary motoneurons (Myers et al. 1986). Non-NMDAR staining was performed with incubation of only anti-GluR2/3 (1:200, Chemicon, Temecula, CA). Fish were washed in phosphate-buffered saline for 3 h every 15 min on a shaker, then incubated in secondary antibody coupled to Alexa568 (goat anti-rabbit 1:1000, Molecular Probes, Eugene, OR) and Alexa488 (goat anti-mouse 1:1000, Molecular Probes) for 4 h at room temperature on a shaker. Fish were washed in phosphate-buffered saline for 3 h every 15 min then mounted in glycerol for viewing. Imaging was done using a Hamamatsu C4742-95 CCD camera

mounted on a Leica DMLFSA epifluorescence microscope with water immersion 20x and 40x objectives. Image capture was done using Openlab software (Improvision, Lexington, MA). Images were contrast enhanced in Openlab.

2.3. Whole-cell Recording

Anesthetized four-to-six dpf zebrafish larvae were pinned through the notochord to Sylgard-lined recording dishes and perfused at 1-2 ml/min with extracellular saline containing (in mM) 134 NaCl, 2.9 KCl, 2.1$CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, 0.001 tetrodotoxin (TTX; Tocris, Avonmouth, UK). A section of skin was removed to allow access to the axial muscles. Following removal of the skin red muscle fibers were gently removed with a micropipette. Red fibers run parallel to the body while white fibers lie at an oblique angle, allowing for visual distinction between the two muscle types (van Raamsdonk et al. 1982). Whole-cell patch-clamp recordings were performed on axial white fibers in voltage-clamp mode (Hamill et al. 1981). For investigation of NMDA receptor properties, Mg^{2+}-free extracellular solution was used. The Mg^{2+}-free superfusate consisted of equimolar NaCl replacing $MgCl_2$. The pipette solution contained (in mM) 130 CsCl, 2 $MgCl_2$, 10 HEPES, 10 EGTA, 2 $CaCl_2$, 4 Na_2ATP, 0.4 LiGTP. Polished pipettes were pulled from thin-walled glass (World Precision Instruments, Sarasota, FL) and had tip resistances of 1-2.5 MΩ, resulting in whole-cell series resistance values of 1.5-5 MΩ. Series resistances were monitored carefully during recordings and abandoned if a change of >15% occurred. All series resistances were compensated by 90-95%, and fibers were

clamped at –65mV throughout all recordings. Whole-cell recordings were performed with an Axopatch 200B and captured with pClamp 8.1 software. Data were sampled at 100 kHz and low pass filtered at 10 kHz. Recording analysis was performed using Axograph software.

Following rupture of the membrane, recordings were allowed to stabilize prior to initiation of recording. Following stabilization, the next 2 min of each recording was used as a measure of the baseline frequency of spontaneous release. Miniature endplate current (mEPC) frequency in the presence of agonists was determined at the visually identified peak frequency, or following 6-6.5 min of agonist perfusion. Spontaneous currents were captured for 30 s surrounding the peak frequency or if no increase in frequency was observed, mEPCs were captured over the last 2 min of the recording. Recordings of mEPCs were analyzed for any kinetic changes associated with agonist perfusion, including, rise-time, decay time, peak amplitude, proportions of small (<400pA) and large (>400pA) events (as determined through amplitude distribution analysis) and frequency of spontaneous release. Kainic acid, AMPA, NMDA and glutamate (Sigma, St. Louis, MO) were used as agonists. Vehicle controls were completed using 0.1% DMSO in saline, as AMPA was dissolved in this solvent.

Agonist specificity was investigated through antagonist application. Antagonists were bath applied for 2 min prior to initiation and during agonist perfusion. The competitive antagonist D-(-)-2-amino-5-phosphonopentanoic acid (AP-5) (Tocris) (50 μM) was used to block NMDA receptor activation (Ali et al. 2000a). Nonspecific (CNQX, 50μM; Sigma) and specific non-NMDAR

antagonists were used to investigate the contribution of AMPARs and KARs. (2S,4R)-4-methylglutamate (Sym 2081, Sym) (Sigma) (10 μM) acts specifically on KARs to desensitize them and prevent their activation (Donevan et al. 1998; Zhou et al. 1997). 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466) (Sigma) (50 μM) was used as a specific antagonist for AMPARs (Cossart et al. 2002; Donevan and Rogawski 1993; Zhang et al. 2002). To investigate potential effects of glutamate, the general glutamate transport blocker DL-threo-β-benzyloxyaspartate (DL-TBOA) (Tocris) (200 μM) was used (Jabaudon et al. 1999; Waagepetersen et al. 2001). D-tubocurarine (10 μM; Sigma) was used to block nAChRs. Testing of agonists and antagonists were performed on separate cells as they could not be held for extended periods of time.

2.4. Local Perfusion

Local perfusion was used in an attempt to directly apply agonist onto the NMJ. To detect the locally applied perfusate, domestic food colouring (5%) was added to the agonist solution. To ensure that the agonist-containing perfusate was removed quickly from the animal a bath flow rate of ~3-4 ml/min was used. The perfusion pipette was placed at the horizontal midline of the larvae with bath flow away from the spinal cord. A cone-shaped flow of perfusate was clearly visible flowing ventrally away from the midline and over the recording pipette. No back-flow over the spinal cord was ever visible.

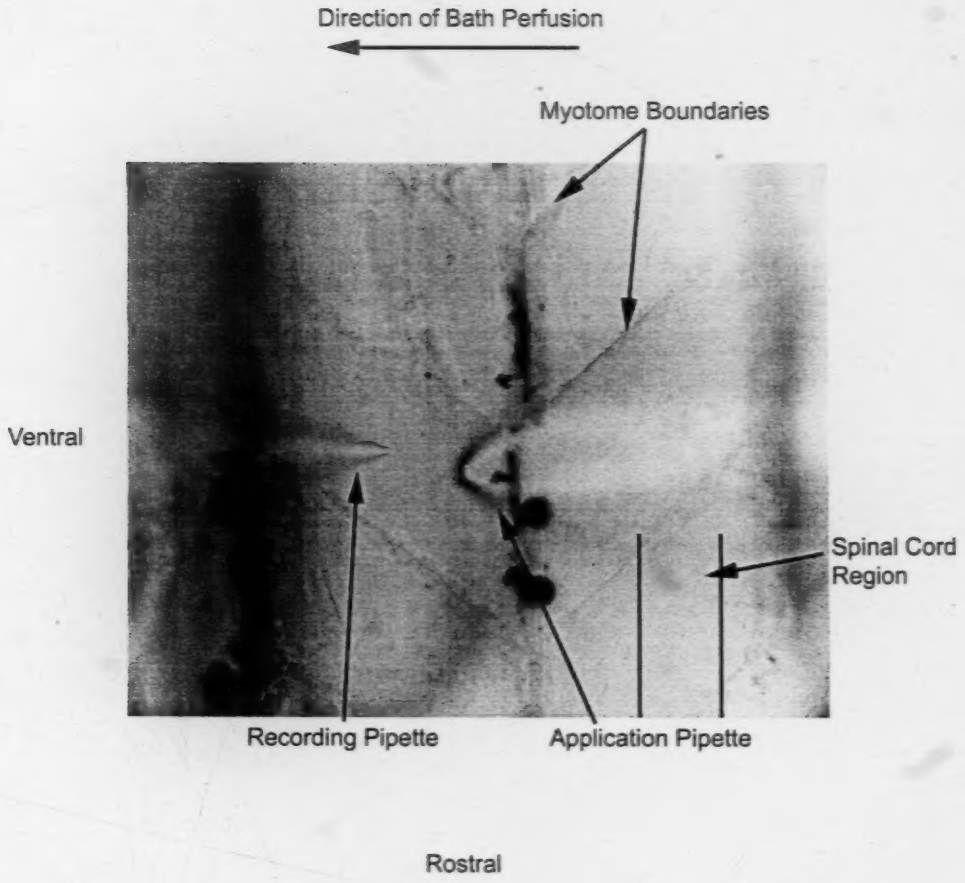


Fig 2. Experimental design used during local perfusion of axial white muscle. The application pipette contains agonist and control solutions that were perfused over the recording region. Bath flow was away from the spinal cord, such that locally applied treatments were removed quickly from the preparation. No local perfusate was ever visible over the spinal cord region.

2.5. Statistical Analysis

Prior to analysis, the normality of the data was tested in SigmaStat. Due to some data sets being non-normal, non-parametric analyses were used throughout. The Mann-Whitney U test was used to compare all treatments. Significance was assigned where P values were less than 0.05. All treatments were replicated 4-8 times. SigmaStat software was used for all statistical analyses and data were plotted using SigmaPlot software.

3. Results

3.1. Immunohistochemistry

Whole-mount preparations of 4-6 dpf zebrafish reveal peripheral staining in the axial musculature with NR2A antibodies (Fig. 3A1,B1). In order to identify which peripheral axons were expressing the NMDAR subunits, double-labeling experiments were performed using the NR2A antibody and the zn-1 antibody that specifically targets primary motoneurons in larval zebrafish (Myers et al. 1986). The NR2A staining appears to be associated with motoneurons, as shown by colocalization with zn-1 antibody staining (Fig. 3A,B). Staining with anti-NR2A reveals what appears to be one common, ventral exit point from the spinal cord, afterwhich axons project to the dorsal, ventral and mid musculatures (Fig. 3A1,B1). The NR2A staining corresponds well with previously published images of primary motoneuron projections (Behra et al. 2002; Eisen and Melançon 2001; Myers et al. 1986; Westerfield et al. 1986). The three primary motoneurons, caudal (CaP), middle (MiP) and rostral (RoP), project to the ventral, dorsal and mid axial musculature, respectively (Myers et al. 1986). NR2A staining colocalizes with zn-1 staining in dorsal, ventral and mid musculature, (Fig. 3A3, B3) suggesting that NMDARs containing the NR2A subunit are associated with the axons of primary motoneurons.

Since glial cells are known to express glutamate receptors (Porter and McCarthy 1996; Zhou and Kimelberg 2001), it was possible that the observed staining was associated with Schwann cells along motoneurons. Immunostains were, therefore, performed using anti-NR2A antibodies on animals at an age at

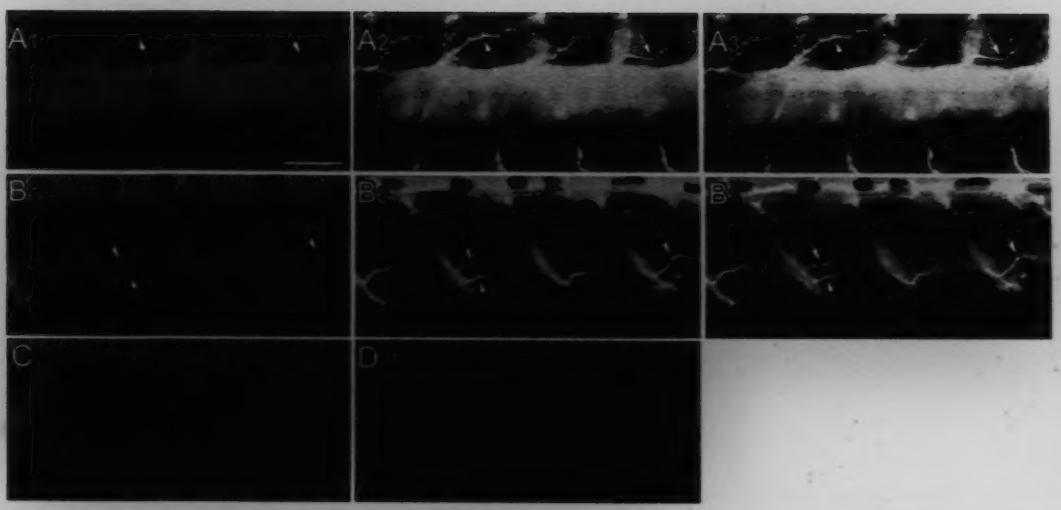


Fig. 3. Immunohistochemistry suggests the presence of NR2A subunits in association with motoneurons. NR2A staining (A_1) in dorsal body segments of a 6 dpf larval zebrafish, with zn-1 staining (A_2) in the same segments. Merged image of dorsal staining shows NR2A staining colocalizes with the zn-1 staining of middle primary motoneuron axons (A_3). NR2A staining (B_1) in ventral body segments of a 6 dpf larval zebrafish, with zn-1 staining (B_2) in the same segments. Merged image of ventral staining shows NR2A staining colocalizes with the zn-1 staining of rostral and caudal primary motoneuron axons (B_3). NR2A staining in 26 hpf embryos is similar to staining in older animals and is associated with motoneuron axons (C). Negative controls lacking primary antibodies show no staining (image captured at identical exposure to treatment images; D). Scale bar is 50 μm (n=6).

which myelination of peripheral axons has not yet occurred (Brosamle and Halpern 2002). NR2A staining in 26 hpf embryos (Fig. 3C) reveals similar staining of projections as seen in older animals, indicating that NR2A subunits are associated with the motoneurons and not Schwann cells.

Negative controls were performed, by excluding primary antibody from tissue incubations. Incubation in secondary antibodies resulted in dull homogenous staining throughout (Fig. 3D). These controls support the specificity of the primary antibodies used.

Immunostains with NR2B antibodies suggested that these subunits might be associated with peripheral axons (Fig. 4A). Although these results were inconclusive, they suggested that NR2B subunits were not associated with zn-1 staining and therefore motoneurons (Fig. 4B, C). An antibody for GluR2/3 subunits resulted in positive staining in the spinal cord (Fig. 4D), but not in peripheral tissues. All immunostains were repeated 4-6 times. iGluR antibodies for NR1, GluR1, GluR5-7 and KA2 provided inconclusive or negative results. In addition an antibody for glutamate was tested, providing inconclusive results. Taken together, these results suggest that NR2A subunits are associated with the axons of developing primary motoneurons.

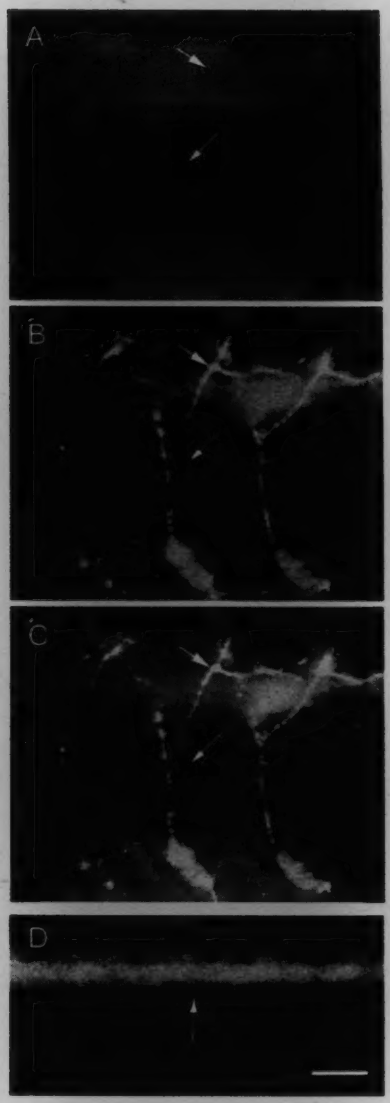


Fig. 4. Immunohistochemistry suggests the presence of NR2B subunits in peripheral neurons. NR2B staining appears in peripheral muscle (A). Zn-1 staining correlates to primary motoneurons (B). Staining with anti-NR2B does not appear to colocalize with zn-1 staining of primary motoneurons (C). GluR2/3 staining appears in the spinal cord (arrow), but not periphery of zebrafish larvae (D). Scale bar is 50 μm (n=4).

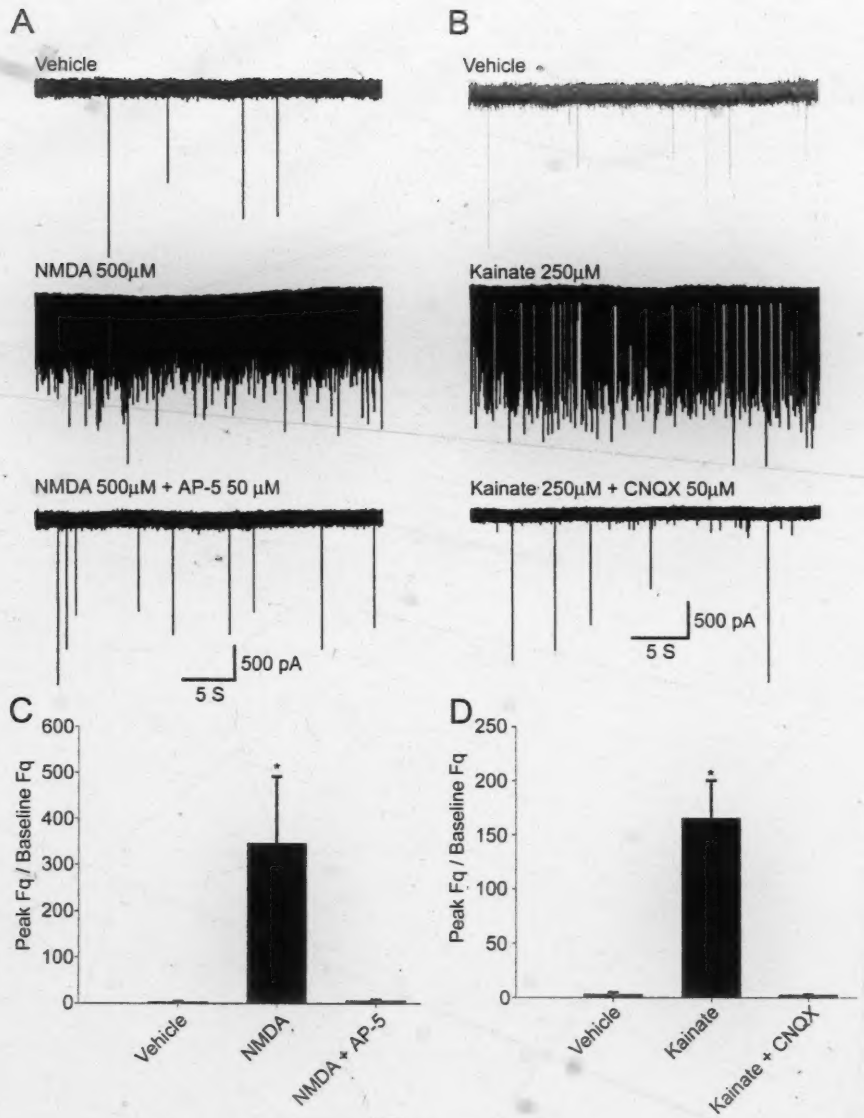
3.2. Local Perfusion

Next, electrophysiology was used to investigate the presence of axonal or presynaptic NMDARs at the NMJ and to confirm the immunohistochemical results. It was hypothesized that activation of NMDARs would depolarize the axons and influence the release of ACh at the endplate. Therefore, mEPCs were recorded from muscle fibers in the presence of glutamate receptor agonists. This was done using a relatively high bath flow-rate (~3 mL/min), flowing from dorsal to ventral over the preparation. The agonist perfusion pipette was placed at, or ventral to the horizontal midline of the fish, also perfusing from dorsal to ventral. Whole-cell patch-clamp recordings were performed on white muscle ventral to the perfusion pipette. With this design, perfusion of only the ventral axial muscle was attained with no back-flow of perfusate over dorsal muscle and the spinal cord. Perfusates may dilute by as much as 10-fold over a 300-500 μm distance (Herreras et al. 1994), and therefore, a relatively high concentration of NMDA was used to ensure activation of axonal or presynaptic receptors. Recordings were taken solely from white muscle, since the immunohistochemistry suggested that NMDARs were associated with primary and not secondary motoneurons. Application of 500 μM NMDA caused a significant ($P<0.05$) rise in the frequency of spontaneous ACh release that was 346.7±145.2 fold that in the presence of saline alone. This increase was blocked by bath application of 50 μM AP-5 (Fig. 5A). Since NMDARs appeared to be present at or near to the NMJ, the possible presence of AMPARs and KARs was investigated. Perfusion with KA (250 μM) induced responses that were significantly ($P<0.05$) greater than saline controls

by 165.5±34.9 fold, and that were prevented with the non-NMDAR antagonist CNQX (50 μM) (Fig. 5B). Graphical representations of raw traces (Fig. 5C, D) illustrate the significant increase in mEPC frequency in the presence of KA and NMDA ($P<0.05$). There was no increase in mEPC frequency in any of the control experiments ($P>0.05$).

3.3. Bath Perfusion

During local perfusion, multiple factors alter agonist concentration at the endplate, such as perfusate flow rate, bath flow rate and distance of the perfusion pipette from the endplate. These properties vary from experiment to experiment and make accurate calculations of agonist concentrations at the NMJ difficult. For this reason, further, more detailed kinetic and pharmacological analyses were performed on recordings taken with the more uniform bath application. Whole-cell voltage-clamp recordings from white axial muscle were performed in the presence of NMDA (100 μM, Fig. 6). Representative traces from these recordings illustrate a large increase in frequency of spontaneous ACh release in the presence of NMDA compared to vehicle control experiments (Fig. 6A). The specificity of the bath-applied agonists was investigated through application of specific NMDA and non-


A
Vehicle
NMDA 500μM
NMDA 500μM + AP-5 50 μM
500 pA
5 S
B
Vehicle
Kainate 250μM
Kainate 250μM + CNQX 50μM
500 pA
5 S
C
Peak Fq / Baseline Fq
600
500
400
300
200
100
0
*
Vehicle
NMDA
NMDA + AP-5
D
Peak Fq / Baseline Fq
250
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150
100
50
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Vehicle
Kainate
Kainate + CNQX

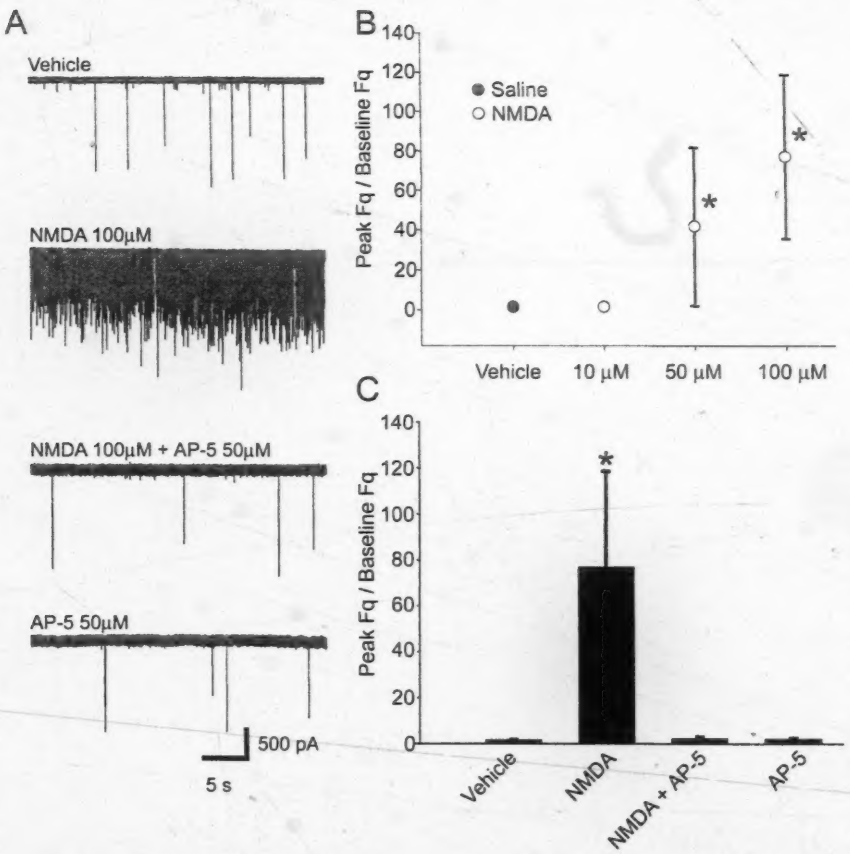


Fig. 6. Frequency of spontaneous ACh release is increased during bath application of 100 μM NMDA. (A) Representative 30 s traces of whole-cell voltage-clamp recordings from axial white muscle. Traces are for bath application of Mg^{2+}-free saline vehicle controls, 100 μM NMDA, 100 μM NMDA + 50 μM AP-5 and 50 μM AP-5. The NMDA-induced increase in spontaneous ACh release is blocked by co-application of 50 μM AP-5. (B) Bath application of 10, 50 and 100 μM NMDA resulted in a partial dose-response curve. (C) Pooled data for spontaneous ACh release (seen in A) with averages and standard error plotted as the normalized frequency. No significant difference in frequency of spontaneous ACh release is apparent in the presence of 100 μM NMDA + 50 μM AP-5 or 50 μM AP-5 alone, when compared to Mg^{2+}-free saline vehicle controls. In the presence of 100 μM NMDA, a significant increase in spontaneous ACh release is observed. Significance indicated by *, $P<0.05$ (n=6-8).

NMDAR antagonists. Bath application of the competitive antagonist to NMDARs, AP-5 (50 μM) prevented the NMDA-induced increases in frequency of spontaneous release (Fig. 6A). Application of 10, 50 and 100 μM NMDA allowed graphing of the dose dependent relationship (Fig. 6B). Bath application of 50 and 100 μM NMDA induced significant increases in the frequency of spontaneous ACh release ($P<0.05$). However, 100 μM NMDA was used for further antagonist studies. Averaged values for individual recordings reveal a significant ($P<0.05$) increase in frequency of spontaneous ACh release in the presence of 100 μM NMDA (Fig. 6C). This increase of 77.0±41.5 fold, that of saline controls, was prevented from occurring in the presence of 50 μM AP-5. No change in mEPC frequency was observed when AP-5 was applied alone ($P>0.05$).

The potential presence of AMPA and KA receptors at the NMJ was also investigated. Representative traces from recordings performed in the presence of each pharmacological application illustrate the AMPA-induced increase in spontaneous ACh release (Fig. 7A). Application of 10, 50 and 100 μM AMPA revealed significant ($P<0.05$) effects at concentrations of 50 and 100 μM (Fig. 7B). Averages of agonist and antagonist applications reveal a significant increase of 114.7±37.1 fold in the frequency of spontaneous ACh release in the presence of 50 μM AMPA compared to vehicle control experiments (Fig. 7C). The non-competitive AMPA receptor-specific antagonist GYKI 52466 (50 μM) prevented the effect of AMPA (50 μM) on spontaneous release ($P<0.05$). The frequency of

spontaneous ACh release was unaltered in the presence of GYKI 52466 alone ($P>0.05$).

Since it was determined that AMPARs are present at the larval zebrafish NMJ, it was of interest to investigate the contribution of KARs to this response. To do this, the specific KAR antagonist Sym 2081 (Sym), that desensitizes KARs at low concentrations, was used (Cossart et al. 2002; Zhou et al. 1997). Representative recordings illustrate the KA-induced increase in spontaneous ACh release compared to controls (Fig. 8A). Concentration of 10, 50 and 100 μM KA were tested. No significant response to 10 μM KA was observed, however, in the presence of 50 and 100 μM KA, significant increases ($P<0.05$) in mEPC frequency were observed (Fig. 8B). The more moderate increase (150.7±56.9 fold) in the presence of 50 μM KA was chosen for further pharmacological investigation. It was found that Sym 2081 (10 μM) had no effect on the KA-induced increase in mEPC frequency (Fig. 8A, C). In addition, Sym 2081 alone had no effect on the frequency of spontaneous release ($P>0.05$). To investigate whether KA was non-specifically activating AMPA receptors, GYKI 52466 (50 μM) was applied in the presence of KA (50 μM). GYKI 52466 prevented the KA-induced increase in frequency of spontaneous ACh release ($P<0.05$) suggesting that KA acts non-specifically to activate peripheral AMPARs.

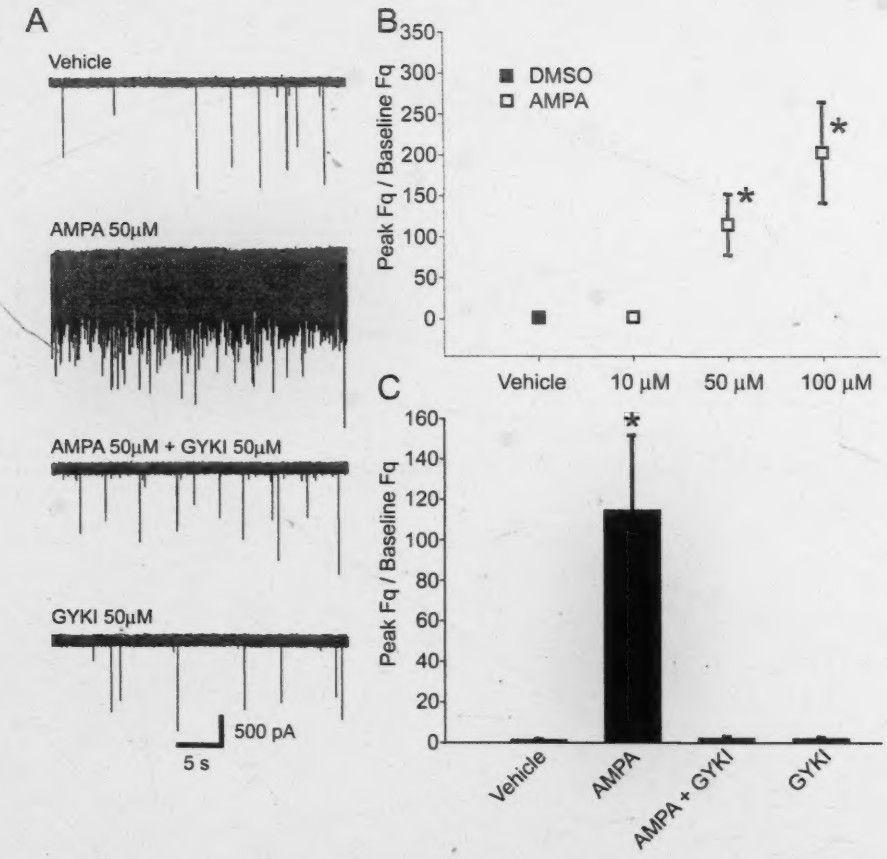
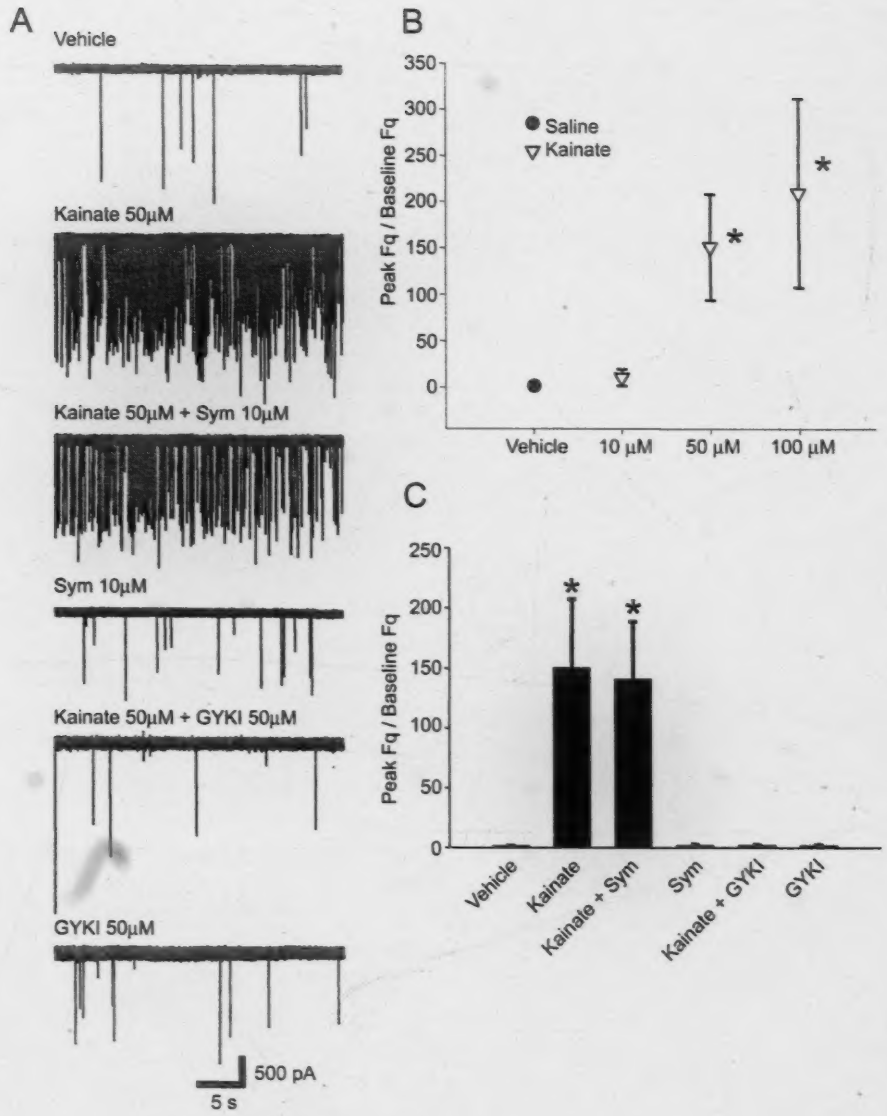


Fig. 7. Frequency of spontaneous ACh release is increased during application of 50 μM AMPA. (A) Representative 30 s traces of whole-cell voltage-clamp recordings from axial white muscle. Traces are for bath application of 0.1% DMSO vehicle controls, 50 μM AMPA, 50 μM AMPA + 50 μM GYKI 52466 and 50 μM GYKI 52466. The AMPA-induced increase in spontaneous ACh release is blocked by co-application of 50 μM GYKI 52466. (B) Bath application of 10, 50 and 100 μM AMPA resulted in a partial dose-response curve. Results show large and significant increases in frequency of spontaneous ACh release at both 50 and 100 μM AMPA. (C) Pooled data for spontaneous ACh release (seen in A) with averages and standard error plotted as the normalized frequency. No significant difference in frequency of spontaneous ACh release is apparent in the presence of 50 μM AMPA + 50 μM GYKI 52466 or 50 μM GYKI 52466 alone, when compared to 0.1% DMSO vehicle controls. In the presence of 50 μM AMPA a significant increase in spontaneous ACh release is observed * indicates $P<0.05$ (n=6-7).

Fig. 8. Frequency of spontaneous ACh release is increased during application of 50 μM KA. (A) Representative 30 s traces of whole-cell voltage-clamp recordings from axial white muscle. Traces are for bath application of saline vehicle controls, 50 μM KA, 50 μM KA + 10 μM Sym 2081, 10 μM Sym 2081, 50 μM KA + 50 μM GYKI 52466 and 50 μM GYKI 52466. (B) Bath application of KA resulted in a significant increase in frequency of spontaneous ACh release. Results show large and significant increases in frequency of spontaneous ACh release at both 50 and 100 μM KA. Increases in mEPC frequency were not blocked by the KAR-specific antagonist Sym 2081. However, the AMPAR-specific antagonist GYKI 52466 prevented the KA-induced increase in frequency of mEPCs. The KA-induced increase in spontaneous ACh release is blocked by co-application of 50 μM GYKI 52466. (C) Statistical representation of spontaneous ACh release (seen in A) with averages and standard error plotted as the normalized frequency. Significant ($P<0.05$) increases in frequency of spontaneous release were observed in the presence of 50 μM KA and KA + 10 μM Sym 2081(denoted with *). However, no significant difference in frequency of spontaneous ACh release was apparent in the presence of 10 μM Sym 2081 alone, 50 μM KA + 50 μM GYKI 52466 or 50 μM GYKI 52466 alone, when compared to saline vehicle controls.


A
Vehicle
Kainate 50μM
Kainate 50μM + Sym 10μM
Sym 10μM
Kainate 50μM + GYKI 50μM
GYKI 50μM
500 pA
5 s
B
Peak Fq / Baseline Fq
350
300
250
200
150
100
50
0
Saline
Kainate
*
*
Vehicle
10 μM
50 μM
100 μM
C
Peak Fq / Baseline Fq
250
200
150
100
50
0
*
*
Vehicle
Kainate
Kainate + Sym
Sym
Kainate + GYKI
GYKI

Although synthetic iGluR agonists allow for pharmacological investigation of individual receptor types, the endogenous neurotransmitter is glutamate. Therefore, glutamate was tested for its ability to affect the mEPC frequency.

Representative traces in the presence of 1 mM glutamate, saline controls and uptake inhibitors (Fig. 9A). In the presence of 1 mM glutamate no significant ($P>0.05$) change in frequency of spontaneous release was observed (Fig. 9). However, treatment with a combination of the general glutamate uptake inhibitor DL-TBOA (200 μM) and 1 mM glutamate, significantly increased ($P<0.05$) mEPC frequency (Fig. 9). During application of 200 μM TBOA alone, no change in mEPC frequency was observed (Fig. 9).

Lastly, to ensure that the mEPCs observed during whole-cell muscle recordings were due strictly to AChR activation, curare was used to block the iGluR agonist-induced rise in mEPC frequency. Curare (10 μM) blocked all mEPCs occurring in the presence of NMDA (100 μM) and KA (50 μM), confirming that the observed mEPCs are due to ACh release and nAChR activation (Fig. 10). Taken together, the agonist and antagonist data for local and bath perfusion, suggest the presence of NMDARs and AMPARs at, or close to the zebrafish NMJ. The observed increase in mEPC frequency that is blocked by curare suggests that glutamate receptors are located presynaptically at the NMJ.

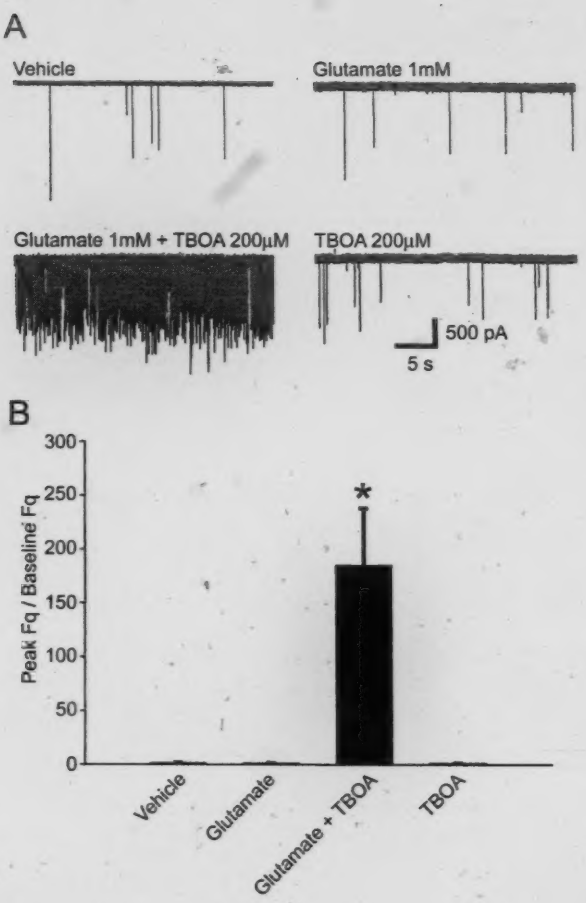


Fig. 9. Glutamate uptake prevents an increase in frequency of spontaneous ACh release. (A) Representative 30 s traces of whole-cell voltage-clamp recordings from axial white muscle. Traces are for bath application of saline vehicle controls, 1 mM glutamate, 1 mM glutamate + 200 µM DL-TBOA and 200 µM DL-TBOA. (B) The application of 1 mM glutamate does not result in a significant increase in the frequency of mEPCs. However, in the presence of the general glutamate uptake inhibitor DL-TBOA, a significant increase in mEPC frequency is observed. Application of 200 µM DL-TBOA alone has no effect on the frequency of spontaneous release. Significance is indicated by * with $P<0.05$ (n=4-6).

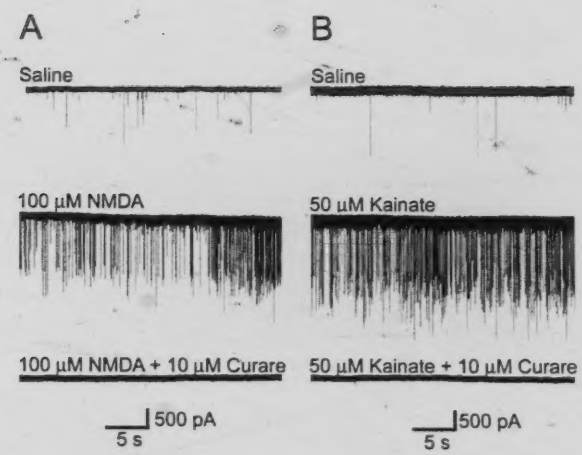


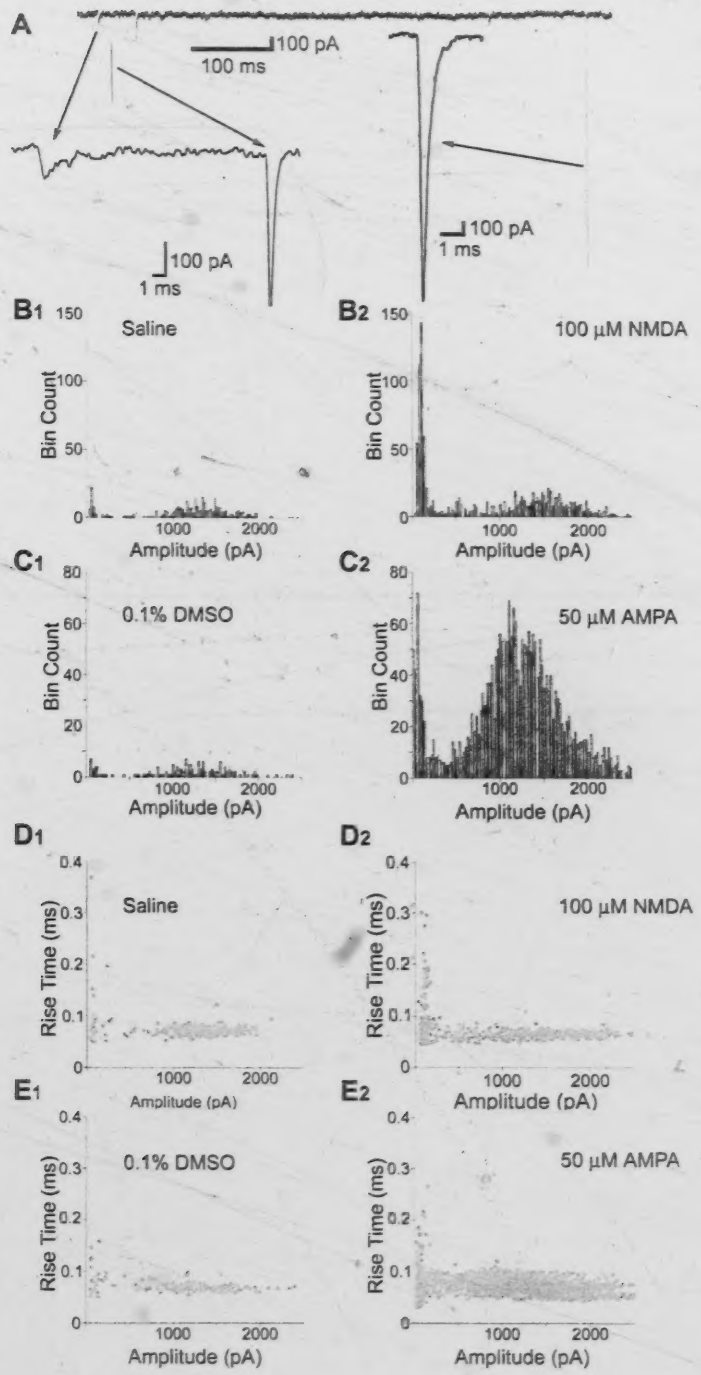
Fig. 10. Curare blocks the iGluR agonist induced increase in mEPC frequency. (A) Application of 10 µM curare in the presence of 100 µM NMDA or (B) 50 µM KA abolishes the agonist induced rise in mEPC frequency (n=3).

3.4. mEPC Kinetics

Bath application of glutamate receptor agonists provided a consistent method of investigating the electrophysiological influence of glutamate receptors on spontaneous ACh release. The present results strongly suggest that the NMDA and AMPARs are located along motoneuron axons or presynaptically at the NMJ. Agonist and antagonist applications suggest the presence of both NMDARs and AMPARs at the NMJ; however, activation of iGluRs might alter the properties of nAChR kinetics in addition to affecting transmitter release. Therefore, mEPC kinetics were investigated in the presence and absence of iGluR agonists.

Analysis of recordings revealed the presence of multiple types of mEPCs (Fig. 11A). Of these pools of events it is likely that large amplitude events (>400 pA) with fast rise times are mEPCs from mature synapses on the muscle being recorded from. A second type of event is of smaller amplitude (<400 pA) and displays relatively long rise times (Fig. 11A). It is probable that these events occur on cells other than the ones being recorded from, and are highly filtered due to electrical coupling (Buss and Drapeau 2000; Nguyen et al. 1999). The third type of mEPC has small amplitudes and fast rise times, and may result from activation of immature synapses, or simply from synapses with fewer numbers of nAChRs. It has previously been reported that two distinct pools of mEPCs can be recorded at the zebrafish NMJ (Nguyen et al. 1999). Nguyen et al. (1999) also found slow and fast rise time events with small amplitudes, although these were not reported as separate pools. Amplitude distributions of all recordings grouped

Fig. 11. Multiple mEPC types are present in whole-cell recordings from axial muscle. (A) A representative 1 s trace recorded in voltage-clamp mode in the presence of 1 μM TTX. Different mEPC types are illustrated in expanded time-scale traces. Note the different rise times and amplitudes. Traces were filtered at 2 kHz for figure clarity. (B) Representative amplitude distributions from recordings in the presence of saline and NMDA (100 μM). (C) A bimodal distribution of mEPC amplitudes is seen in the presence of 0.1% DMSO and AMPA (50 μM). (D) Rise time plotted against amplitude of mEPCs illustrates the different pools of mEPCs in the presence of saline and NMDA. (E) Similar rise time vs. amplitude plots are seen in the presence of 0.1% DMSO and AMPA. Small amplitude events range in rise time where as mEPCs above 400 pA have consistently fast rise times.


A
100 pA
100 ms
100 pA
1 ms
100 pA
1 ms
B1
Saline
B2
100 μM NMDA
Bin Count
Amplitude (pA)
C1
0.1% DMSO
C2
50 μM AMPA
D1
Saline
D2
100 μM NMDA
Rise Time (ms)
E1
0.1% DMSO
E2
50 μM AMPA

together illustrate a clearly bimodal distribution of mEPCs (Fig. 11B, C) that is not altered in the presence of AMPA or NMDA. It is evident that there is a pool of events that have mean amplitudes of about 150 pA with the division between event pools around 400 pA (Fig. 11B, C). The large (>400 pA) amplitude events have a normal distribution with a mean value of 1422 ± 110 pA (n=7 experiments) in the presence of saline that is almost identical to the average amplitude of mEPCs in the presence of NMDA (1433 ± 139 pA; n=8 experiments). The average amplitude of large events is 1455 ± 34 pA and 1340 ± 140 pA in the presence of 0.1% DMSO and AMPA respectively (n=6 experiments). Neither of these pairs of values are significantly different. Rise times vs. amplitude plots for grouped experiments clearly illustrate the different pools of mEPCs (Fig. 11D, E). Small amplitude events range in rise times from 0.05 ms to ~ 0.4 ms, while larger amplitude events have fast rise times between 0.05 and 0.09 ms.

Although there was no apparent change in mEPC amplitude of the large events in the presence of glutamate receptor agonists, it was possible that the pharmacological treatments were altering the nAChR kinetics. Analysis of normalized data in the presence of agonists revealed no significant difference in the data for rise time, decay time, peak amplitude or proportion of large (>400 pA) amplitude events in the presence of NMDA, AMPA, KA or glutamate (Fig. 12).

Small mEPCs (<400 pA) with rise times between 0.05 and 0.1 ms were also analysed. It was important to isolate fast mEPCs (<0.1 ms), to ensure that

these events were occurring on fibers from which recordings were taken. When these events were analysed, no significant differences were observed for rise time, τ or peak amplitude ($P>0.05$; Fig. 13).

The lack of difference in the above properties indicates that the change in spontaneous ACh release has no effect on postsynaptic receptor kinetics, suggesting that the only action of glutamate receptor agonists is at the presynaptic terminal or presynaptic axon. Furthermore, these results also suggest that since the proportion of large events does not change in the presence of the iGluR agonists, then neither does the proportion of small events since these are reciprocal values.

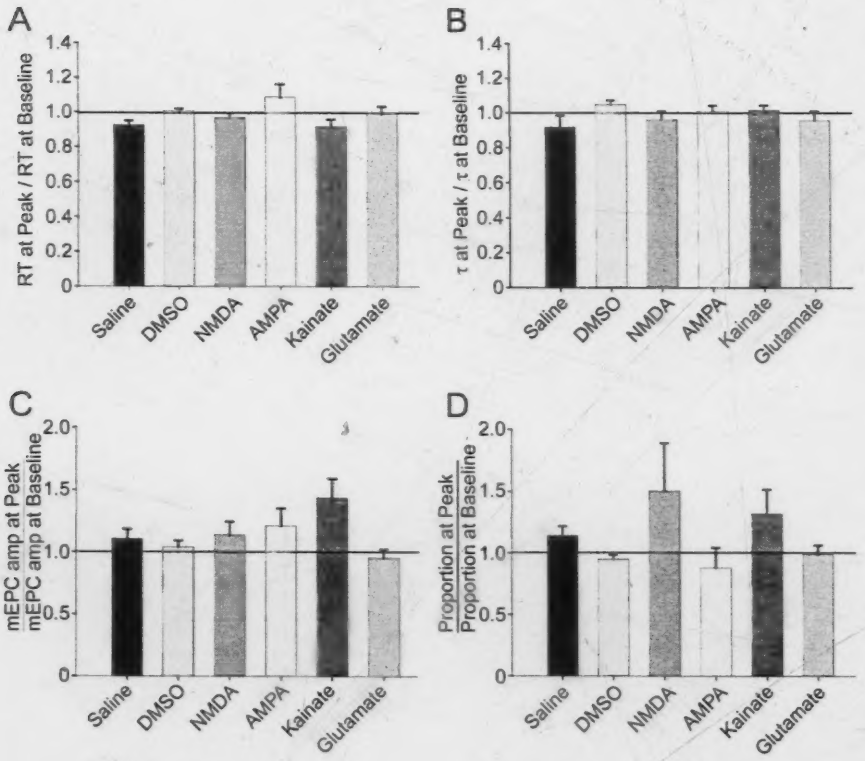


Fig. 12. Comparison of rise time (RT), decay time (τ), mEPC amplitude (amp) and proportion of large (>400pA) events during application of NMDA (100 μM), AMPA (50 μM), KA (50 μM) and glutamate (1 mM) + TBOA (200 μM). (A) No difference in normalized rise time was observed. (B) No difference in normalized τ was observable in the presence of any of the agonists. (C) Average mEPC amplitude of large events normalized to baseline is unaffected by the application of any of the agonist treatments. (D) The proportion of large events during agonist application is shown as a ratio of the proportion of large events during baseline. No significant difference in the proportion of large events was observed for any of the agonist treatments ($P>0.05$; n=4-8).

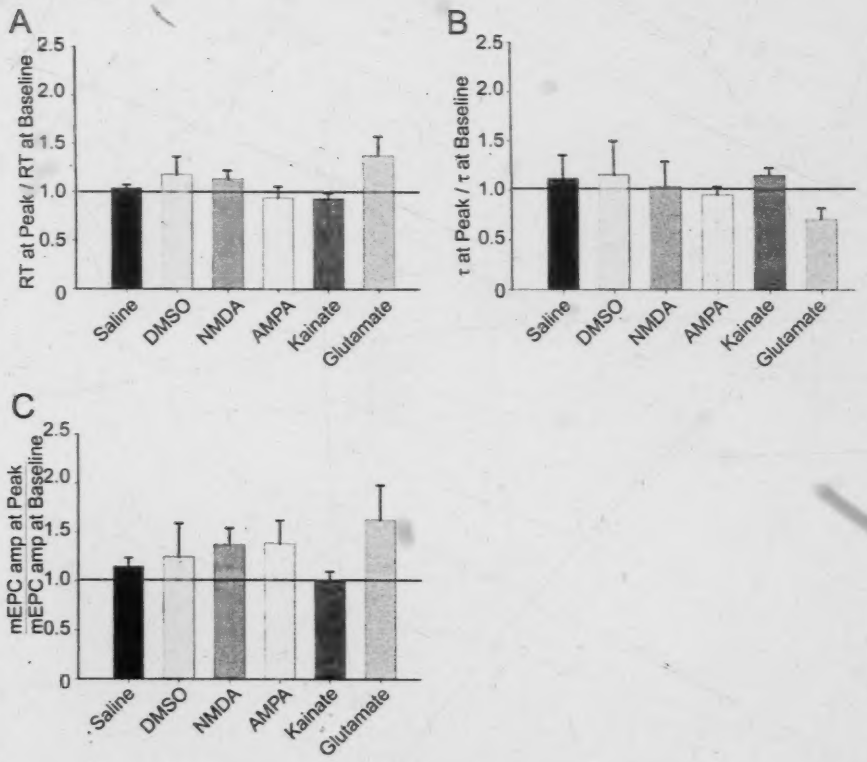


Fig. 13. Comparison of rise time (RT), decay time (τ), and mEPC amplitude (amp) for small (<400pA) events during application of NMDA (100 μM), AMPA (50 μM), KA (50 μM) and glutamate (1 mM) + TBOA (200 μM). (A) No difference in normalized rise time was observed. (B) No difference in normalized τ was observable in the presence of any of the agonists. (C) Average mEPC amplitude of small events normalized to baseline is unaffected by the application of any of the agonist treatments (n=4-8).

4. Discussion

The combination of immunohistochemistry and electrophysiology strongly suggest the presence of iGluRs at the developing zebrafish NMJ. This is the first study to show a specific NR2 subunit associated with axons of motoneurons in the periphery. In addition, this is the first suggestion that AMPARs may be associated with the axons of motoneurons in the periphery. The use of specific non-NMDAR antagonists provides support for the presence of AMPARs but not KARs. Finally, these results also suggest that the NMDA receptors may be localized to primary, but not secondary motoneurons. Although this is the first report of this occurring in zebrafish, iGluRs have been suggested to be present at the NMJ of *Xenopus* and rat by a number of different groups (Barthelemy-Requin et al. 2000; Fu et al. 1995; Grozdanovic and Gossrau 1998; Liou et al. 1996; Malomouzh et al. 2003; Waerhaug and Ottersen 1993). When taken together, these results strongly suggest the presence of iGluRs in association with motoneuron axons outside the CNS. Furthermore, application of curare in the presence of iGluR agonists suggests that these receptors are not associated with muscle fibers.

4.1. iGluRs at the NMJ

Data presented here suggest the presence of NMDARs in association with the axons of motoneurons. Although antibody staining appears all along axon tracts, electrophysiological results support the presence of axonal or presynaptic NMDARs. Also, receptors may be associated with axons in an en passant

manner or with presynaptic terminals. From either location modulation of synaptic function may occur (Cochilla and Alford 1997). The pattern of NR2 staining may not directly indicate the presence of presynaptic receptors, but does support the presence of these receptors outside motoneuron somata. Evidence for axonal transport is another possible explanation for the observed staining along axons. Liu and colleagues (1994) report finding retrograde transport of presynaptic receptors for degradation. These NMDARs were synthesized at the terminal and were transported to somata for degradation during normal receptor cycling. The possibility that axonal receptors could modulate synaptic function, or that axonal transport may be occurring, could both explain the homogenous staining observed along motoneuron axons.

Since staining in 26 hpf embryos was similar to what was present at 4-6 dpf, NR2A subunits must be associated with motoneuron axons and not glia. Although glial cell precursors are present in embryonic zebrafish (Gilmour et al. 2002), and may be associated with axon pathfinding and motoneuron differentiation (Gilmour et al. 2002; Park et al. 2002) it has been suggested that myelination does not occur until about 4 dpf (Brosamle and Halpern 2002).

NR2 subunits are a major source of diversity in NMDARs as they alter many properties of these receptors. One of these differences includes voltage-dependence and Mg^{2+} sensitivity. Other differences in NR2 subunits include responses to both glycine (Regalado et al. 2001) and glutamate (Chen et al. 2001), and mechanisms of modulation (Krupp et al. 1996). It has also been suggested that differences in surface expression pattern occur between subunits.

Barria and Malinow (2002) suggest that NR2A subunits require ligand binding to be inserted into the membrane, while NR2B subunits do not. This may provide a mechanism that allows for selective surface expression of axonal NMDARs in zebrafish motoneurons. NR2A subunits also have shorter decay times (Cathala et al. 2000), which may be important for faster on and off responses to changes in ligand availability. Also, NR2A-containing receptors are more permeable to Ca^{2+}, which has been shown to be functionally important when NMDARs are the only Ca^{2+}-permeable receptor at a synapse (Lei and McBain 2002). Any of the above subunit-specific characteristics could be important for the selective expression of NR2A subunits at the NMJ.

Preliminary data with NR2B subunit staining suggested their presence in association with peripheral axons. However, colabels with zn-1 suggested that NR2B subunits were not associated with motoneurons, but perhaps another peripheral neuron type. It is possible that these receptors were associated with dorsal root ganglia, as NMDARs have previously been reported to be present on presynaptic terminals of these neurons (Liu et al. 1994; Marvizon et al. 1997). Due to the inconsistency of these immunolabels these experiments were abandoned. It is, however, also possible that NR2C or NR2D subunits are also present in association with peripheral axons. Although many different antibodies for NMDAR and non-NMDAR subunits were tested, few useable results were obtained. One result for non-NMDAR subunits, suggested the presence of GluR2/3 subunits in the CNS, but not in the PNS.

Electrophysiological investigation of non-NMDARs suggests the presence of AMPARs, but not KARs at the NMJ. This is suggested through the use of specific AMPAR and KAR antagonists (GYKI 52466 and Sym 2801 respectively). Since EC_{50} values for activation of AMPARs by KA have been reported to be between 56 and 150 μM (Lambolez et al. 1991; Lazzaro et al. 2002; Jayaraman 1998; Nakanishi et al. 1990; Patneau et al. 1993) the concentrations of KA that we used were potentially activating both AMPA and KARs. Prevention of the KA-induced increase in mEPC frequency by GYKI 52466 supports indiscriminate AMPAR activation by KA.

If AMPARs are present at NMJs, their action may be different from that of NMDARs. AMPARs are known to have fast kinetics that may lead to removal of the voltage-dependent Mg^{2+} block on NMDARs (Hollmann and Heineman 1994). At NMJs, these receptors may have a similar role, allowing for activation of NMDARs. However, it is also possible that AMPARs are simply another source of Ca^{2+} entry into presynaptic terminals. It is known that all subunit compositions of AMPARs that do not have the GluR2 subunit are permeable to Ca^{2+} (Hollmann and Heineman 1994). Preliminary data from GluR2/3 antibody staining suggested that these subunits were not present on motoneuron axons. However, these experiments were inconclusive and it was not determined which subunit was targeted with this antibody. Another possibility is that AMPARs at the NMJ are linked to second messenger molecules. It has been shown that ionotropic AMPARs can be linked to G-proteins, adding another level of potential modulation (Kawai and Sterling 1999). AMPARs have the potential to add

multiple levels of synaptic modulation to any system. It would be an interesting and important task to determine their exact action at NMJs.

Although it appears that NMDARs are only associated with primary motoneurons, increases in the frequency of mEPCs occur for both large and small amplitude events. Perhaps different amplitude events arise from activity occurring on different branches of the same primary motoneuron. It is possible that endplates associated with different branches have reached different points of maturation, resulting in different sized mEPCs. Assuming that large mEPCs are from primary motoneurons while small mEPCs are from secondary motoneurons, it is also possible that secondary motoneurons express iGluRs as well, but express subunits other than NR2A.

Whatever the exact location of iGluRs at NMJs, it appears that both NMDARs and AMPARs are present there. Their subunit composition and function has yet to be determined, however, they are possibly involved in enhancement of synaptic release or raising presynaptic Ca^{2+}.

4.2. Glutamate at NMJs

Recently, the presence of glutamate at the NMJ has been suggested in mammalian preparations (Barthelemy-Requin et al. 2000; Waerhaug and Ottersen 1993). There is also evidence that glutamate and ACh can be coreleased from vertebrate synaptosomes (Vyas and Bradford 1987). It does, however, seem most likely that glial cells surrounding the NMJ are involved in glutamate uptake and release. Although many types of glutamate transporters

are probably present at the NMJ, previously published literature suggests that glial transporters are primarily responsible for glutamate clearance (Bergles and Jahr 1998; Danbolt 2001). The lack of effect in the presence of glutamate alone suggests that glutamate is either being taken up or being metabolized (Danbolt 2001; Matthews et al. 2000). This strongly suggests that glutamate is being removed from the site of action at a rate that prevents activation of iGluRs. Rapid removal of glutamate reflects the tight regulation of this neurotransmitter at the synaptic cleft to prevent excitotoxicity and diffusion (Asztely et al. 1997).

It is also possible that glutamate is released from glial cells surrounding the NMJ. Although this has not been reported to occur at the NMJ, glial cells of the CNS are thought to have vesicular machinery (Calegari et al. 1999) and are able to release glutamate in a non-pathological, vesicular manner (Araque et al. 2000; Pasti et al. 2001). Also, Waerhaug and Ottersen (1993) reported the presence of glutamate in perisynaptic Schwann cells at rat NMJs. This release could be mediated through a feedback mechanism involving signaling molecules like NO or perhaps through muscarinic AChRs which are known to be present on glial cells surrounding the NMJ (Robitaille et al. 1997).

Although no direct evidence for glutamate at zebrafish NMJs was obtained (as immunostaining for glutamate was inconclusive), the presence of iGluRs suggests that glutamate is there. Glutamate may be released from either motoneuron terminals or perisynaptic Schwann cells. In some neurons, glutamate may be coreleased with dopamine (Krebs et al. 1991) and possibly ACh (Vyas and Bradford 1987). Its possible release at zebrafish NMJs is

therefore not a novel suggestion. Glutamate at vertebrate NMJs is not surprising, as it is the main neurotransmitter at invertebrate NMJs (Keshishian et al. 1996) and may have remained over the course of evolution, simply switching to a modulatory role. It is foreseeable that modulation of NMJs could occur through release of a transmitter other than ACh as similar mechanisms are thought to occur in other neurons with other neurotransmitters (Sulzer and Rayport 2000).

4.3. The Role of iGluRs at the NMJ

Glutamate is the major excitatory neurotransmitter in the vertebrate CNS. During development, glutamate and its receptors have many important roles for outgrowth (Metzger et al. 1998), plasticity (Grant and O'Dell 2001; Luscher et al. 2000) and synaptic strengthening (Gu et al. 1996). Previously reported work on glutamate receptors at the NMJ has suggested that Ca^{2+} may play roles in developmental changes in the presynaptic terminal (Fu et al. 1995). But aside from this, iGluRs may also alter muscle contraction (Koyuncuoglu et al. 1998) or aid in strengthening postsynaptic components of the NMJ. Cochilla and Alford (1999) report a decrease in synaptic failure rate at reticulospinal synapses due to presynaptic NMDARs. A similar mechanism could be taking place at zebrafish NMJs. This may provide a mechanism that ensures strong synaptic transmission during escape responses or, while synapses are still maturing. It is known that pruning of motoneurons is activity dependent and that postsynaptic nAChR clustering is also dependent on the presence of the motoneuron (Luo et al. 2002;

Sanes and Lichtman 1999). If iGluRs increase ACh release and subsequent activity at the developing NMJ then they could play a role in both pre- and post-synaptic modifications.

Ionotropic glutamate receptors at the NMJ may be replaced with mGluRs at a later developmental stage (Liou et al. 1996). This may reflect a developmental change in requirements for glutamate at the NMJ. Initial excitatory actions of glutamate switching to inhibitory ones later in life may occur to strengthen the synapse early then switch to a modulatory role. Strengthening or other glutamate-mediated actions could occur through a Ca^{2+}-mediated mechanism or via another pathway such as NO signaling (Grozdanovic and Gossrau 1998; Malomouzh et al. 2003; Pinard et al. 2002).

Taken together, these results suggest the presence of iGluRs at the larval zebrafish NMJ. Electrophysiology and kinetic analysis of mEPCs suggest that iGluRs do not directly modulate nAChR kinetics. However, it is possible that increased Ca^{2+} in both the pre- and post-synaptic terminals could have a role in the development of the NMJ. Both pre- and post-synaptic components require Ca^{2+}-dependent changes following motoneuron contact that could be facilitated by iGluRs.

This work on iGluRs at the NMJ is only an initial characterization. Further work in this area should focus on investigating the function of iGluRs at the NMJ. To aid understanding the role of iGluRs at NMJs, determining which NMDAR and non-NMDAR subunits are present, would be of value. Knowledge of subunit expression could provide insight into function. Changes in expression may also

occur over development. This is another potentially interesting aspect of glutamate receptors at the NMJ.

5. Conclusion

Results strongly support the presence of iGluRs at the NMJ of larval zebrafish (Fig. 12). Although immunohistochemical staining did not allow for the specific localization of NR2A subunits to the presynaptic terminal, there was the appearance of staining along motoneuron axons. This suggests that NR2A subunits are either expressed along the axons or are being transported to or from terminals. Local perfusion over axial muscle during patch-clamp recordings also supports the hypothesized presence of iGluRs at the NMJ. Although these experiments were not definitive, they are strongly suggestive. Application of agonists and antagonists suggest the presence of NMDARs and AMPARs at NMJs. However, application of specific KAR antagonists, suggest the absence of these receptors. Finally, the functional relevance of iGluRs at larval NMJs was not determined. However, pharmacological applications revealed a role for both NMDARs and AMPARs in spontaneous ACh release. These iGluRs may be involved in development or strengthening of embryonic and larval synapses.

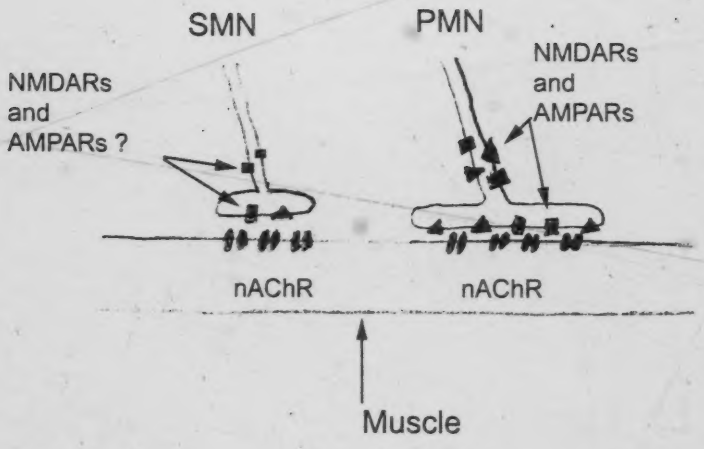


Fig. 14. Schematic of proposed location of iGluRs at NMJs. It is suggested that primary motoneurons (PMNs) express NMDARs and AMPARs on presynaptic or axonal regions at axial NMJs. It is also possible that secondary motoneurons (SMNs) express iGluRs.

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